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Regulation of α-Smooth Muscle Actin Expression in Granulation Tissue Myofibroblasts Is Dependent on the Intronic CArG Element and the Transforming Growth Factor-β1 Control Element

James J. Tomasek,* Joel McRae,*
Gary K. Owens,† and Carol J. Haaksma*

From the Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; and the Department of Molecular Physiology and Biological Physics,† University of Virginia, Charlottesville, Virginia*

Myofibroblasts are specialized contractile fibroblasts that are critical in wound closure and tissue contracture. Generation of contractile force is correlated with the expression of α-smooth muscle actin (α-SMA); however, little is known regarding molecular mechanisms that control activation of α-SMA in myofibroblasts in granulation tissue. The aims of the present studies were to identify sufficient promoter regions required for α-SMA expression in myofibroblasts *in vivo* and to determine whether activation of α-SMA expression in myofibroblasts *in vivo* is dependent on an intronic CArG [CC(A/T)$_6$GG] and a transforming growth factor-β1 control element (TCE) that are required for α-SMA expression in smooth muscle cells. A Lac Z transgene construct from −2600 through the first intron was expressed in myofibroblasts within granulation tissue of cutaneous wounds in a pattern that closely mimicked endogenous α-SMA expression. Mutation of either the intronic CArG element or the TCE completely inhibited transgene expression in myofibroblasts in granulation tissue and responsiveness to transforming growth factor-β1 in cultured transgenic fibroblasts. These same elements were also critical in regulating α-SMA expression during skeletal muscle repair but not during skeletal muscle development. Taken together, these results provide the first *in vivo* evidence for the importance of the intronic CArG and TCE *cis*-elements in the regulation of α-SMA expression in myofibroblasts in granulation tissue. ***(Am J Pathol 2005, 166:1343–1351)***

Myofibroblasts are specialized contractile fibroblasts that play a critical role in generating the contractile force responsible for wound closure and pathological contractures.[1,2] These cells are characterized by the acquisition of a contractile phenotype and the expression of α-smooth muscle actin (α-SMA),[3–5] which is correlated with generation of contractile force.[6,7] An understanding of the regulation of expression of α-SMA in myofibroblasts will be important in controlling the formation and function of myofibroblasts in wound healing and pathological contractures. A major goal of our laboratory has been to elucidate control processes that regulate myofibroblast differentiation by identifying mechanisms that control expression of α-SMA and other contractile cytoskeletal proteins.

α-SMA is transiently expressed in the myocardium and skeletal muscle during the development of the embryo and is highly restricted in adult animals to smooth muscle cells (SMCs);[8,9] however, it is also expressed in myofibroblasts during wound healing and in a very limited number of adult tissues.[1,2] Myofibroblast differentiation *in vivo* has been proposed to be dependent on a number of local environmental cues, including local production of growth factors such as transforming growth factor (TGF)-β1,[5,10] extracellular matrix-integrin interactions,[11,12] and mechanical stress.[1,13] A number of *cis*-elements and *trans*-acting factors have been described that regulate gene expression of α-SMA in SMCs.[14] In contrast, the mechanisms that regulate the expression of α-SMA and other SM-specific cytoskeletal proteins in myofibroblasts are only poorly understood, and, to date, no studies have examined *cis*-elements that may function to regulate α-SMA expression in myofibroblasts *in vivo* within granu-

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Address reprint requests to James J. Tomasek, Ph.D., Department of Cell Biology, BMSB 553, The University of Oklahoma–Health Sciences Center, 940 Stanton L. Young Blvd., Oklahoma City, OK 73104. E-mail: james-tomasek@ouhsc.edu.

lation tissue. By use of a transgenic approach it has been demonstrated in SMCs *in vivo* that a α-SMA promoter construct from −2600 through the first intron (p2600Int) is the sufficient promoter sequence required to drive high-level expression in a manner that mimics the endogenous α-SMA gene.[15] In this study we examined whether a similar α-SMA promoter construct from −2600 through the first intron will be sufficient to drive expression in myofibroblasts in granulation tissue.

The α-SMA promoter, similar to promoters of other SM cytoskeletal proteins, contains multiple CArG elements and a TGF-β1 control element (TCE).[14,16] The three CArG elements, designated CArG A at −62, CArG B at −112, and an intronic CArG at +1001, are completely conserved in all species in which the α-SMA promoter has been cloned and have been shown to be required for full promoter activity in cultured SMCs and in SMCs in transgenic mice.[15–17] In transgenic mice mutation of the CArG B element in the p2600Int promoter completely inhibited expression in all cell types, whereas mutation of the intronic CArG abolished expression in SMCs, but not in developing skeletal and cardiac muscle.[15] These results suggest that the intronic CArG may act as a SMC-specific enhancer-like element. Similarly, the TCE, present at −42 bp in the α-SMA promoter, has been demonstrated to confer TGF-β1 responsiveness to this promoter in both cultured SMCs and fibroblasts.[17,18] However, no studies to date have examined the role of the intronic CArG element or the TCE in regulation of α-SMA expression in myofibroblasts *in vivo* in granulation tissue.

Serum response factor (SRF) is a relatively ubiquitous transcription factor that can bind to all three CArG elements in the α-SMA promoter and appears to be essential for the expression of α-SMA.[15,19,20] Blockade of SRF activity with a dominant-negative SRF mutant can prevent expression of α-SMA.[21] Recently, it has been demonstrated that SRF expression is increased and there is increased nuclear staining for SRF in myofibroblasts in rat lung tissue using a bleomycin-induced pulmonary fibrosis model.[22] Whether this also occurs during myofibroblast differentiation in granulation tissue has not been examined.

The aims of the present study were to address the following questions: 1) is the p2600Int promoter sequence, which is sufficient to direct α-SMA expression in SMCs, sufficient to drive expression in myofibroblasts in granulation tissue? 2) Is regulation of α-SMA expression in myofibroblasts similar to SMCs or similar to developing cardiac and skeletal muscle with regard to the intronic CArG element? 3) Are both the intronic CArG element and the TCE critical for expression of α-SMA in myofibroblasts in granulation tissue and for responsiveness to TGF-β1? The identification of myofibroblast-specific *cis*-elements critical for α-SMA expression is necessary to identify specific cytokines and other factors present within the extracellular milieu of granulation tissue that regulate myofibroblast differentiation and to achieve our long-term goal of using gene therapy to regulate myofibroblast formation and function and thereby manipulate wound closure and pathological contractures.

Materials and Methods

Construction of Rat α-SMA Lac Z Reporters and Generation and Analysis of Transgenic Mice

The reporter constructs, p2600Int/Lac Z, mutated intronic CArG p2600Int/Lac Z, and mutated TCE p2600Int/Lac Z, were generated as previously described.[15,23] The generation of the transgenic mice carrying the p2600Int/Lac Z construct and the mutated TCE p2600Int/Lac Z construct that were analyzed in this article have been previously described.[15,23] The mutated intronic CArG p2600Int/Lac Z construct was prepared for transgenic injection by removal of pUC19 backbone sequences by *Not*I/*Eco*RI digestion and subsequent agarose gel purification of the linearized promoter/Lac Z fragment. Transgenic mice were generated with standard methods within the transgenic core facility at the Oklahoma Medical Research Foundation, Oklahoma City, OK. Mice were analyzed by establishing founder lines that allowed more detailed analysis of transgene expression in neonates and adult animals. Transgene presence was analyzed by polymerase chain reaction with genomic DNA purified from tail clips or ear punches according to the method of Vernet and colleagues.[24] To examine for transgene expression in tissues mice were euthanized by CO_2 asphyxiation (adults) or decapitation (neonates) and tissues stained for β-galactosidase activity as previously described.[25,26] Briefly, tissues were rinsed in cold phosphate-buffered saline (PBS), fixed in 2% paraformaldehyde in 0.1 mol/L PIPES buffer, pH 6.9, for 15 minutes at 4°C and rinsed three times for 5 minutes in PBS. They were stained in β-galactosidase staining solution (5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 2 mmol/L magnesium chloride, 20 μl of 10% Ipegal, 0.01% sodium deoxycholate, 0.1% X-gal) at 30°C overnight in the dark. Tissues were either observed as whole mounts or dehydrated, paraffin-embedded, and sectioned. All animal procedures used in these studies were reviewed and approved by the University of Oklahoma–Health Sciences Center or the University of Virginia Institutional Animal Care and Use Committee.

Preparation of Granulation Tissue from Cutaneous Wounds on Mice

Male mice were wounded between 6 to 8 weeks of age. General anesthesia was induced by intraperitoneal injection of avertin with 0.015 to 0.017 ml of 2.5% avertin per g of body weight.[27] The back was shaved with clippers and cleansed with 70% ethanol. Two circular, overlapping, full-thickness skin wounds were made on the midline by a sterile 3-mm biopsy punch. The excisions extended through the panniculus carnosus to muscular fascia. The wounds were left uncovered (air-exposed) throughout the experimental period. The wounds were excised at time points between 7 to 21 days. The animal was euthanized by CO_2 asphyxiation and the wound plus 4 mm of surrounding dermis was excised. The tissue was placed on a piece of filter paper in a 35-mm Petri dish to maintain its shape and prepared for examination of transgene expression as described above. Paraffin sections were cut at 4 to 6 μm and placed on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA), deparaffinized in xylene, and rehydrated. The morphology of wound tissue was evaluated in hematoxylin and eosin-stained sections by light microscopy. Transgene activity was evaluated by examining adjacent unstained sections by light microscopy for blue staining resulting from β-galactosidase activity.

Immunohistochemical Staining for α-SMA and SRF Expression

Paraffin sections were deparaffinized and rehydrated sections were washed in PBS and blocked in a 1:10 solution of normal goat serum in PBS for 30 minutes. For α-SMA immunohistochemistry sections were incubated with a monoclonal mouse anti-α-SMA antibody conjugated to fluorescein (1:250 dilution, F3777; Sigma-Aldrich, St. Louis, MO), followed by rabbit anti-fluorescein antibody (1:750 dilution, A889; Molecular Probes, Eugene, OR) and subsequently incubated with biotinylated goat anti-rabbit IgG (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). The sections were then incubated with diluted ABC-alkaline phosphatase complex (Vector Laboratories) and color developed in the presence of Vector red alkaline phosphatase substrate (Vector Laboratories). Control sections were treated identically, but without the primary antibody, and did not demonstrate any positive staining. For SRF immunohistochemistry sections were incubated with a rabbit anti-SRF antibody (1:50 dilution, SC-335; Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently visualized as described above except color was developed in the presence of Vector black alkaline phosphatase substrate.

Preparation of Mouse Embryo Fibroblasts (MEFs) and Reporter Gene Assays

MEFs were prepared as previously described.[27] Briefly, mouse embryos 12.5 to 14.5 PC were obtained and the limbs, internal organs, and upper part of the head containing the brain removed. The embryos were finely minced and digested sequentially with trypsin-ethylenediaminetetraacetic acid until only indigestible bone and cartilage remained in the tube. Digestion was stopped with Dulbecco's modified Eagle's medium (Invitrogen-Gibco, Carlsbad, CA) and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and cells were cultured in growth media comprised of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% antibiotic-antimycotic solution (Invitrogen-Gibco), and 1 mmol/L sodium pyruvate (Sigma-Aldrich). The cells were plated in 100-mm tissue culture dishes each containing 10 ml of growth media, ~1.5 dishes per embryo dissected. The cells were grown to confluency and passed or cryopreserved.

For reporter gene assays, MEFs were isolated as described above from mice carrying the p2600Int/Lac Z transgene or the mutated intronic CArG or mutated TCE. MEFs were grown in growth media at 37°C in a humidified atmosphere of 5% CO_2 with media changes every 2 days and passed at 90% confluency or below. MEFs were used up to passage 4. TGF-β1-treated cultures received 1 ng/ml of TGF-β1 (human recombinant, T7039; Sigma) and fresh TGF-β1 was added at each media change or every 48 hours for up to 96 hours. After 24, 48, 72, or 96 hours in culture the medium was aspirated, the cell layer washed twice with Ca-Mg-free PBS and then lysed directly in the dish with reporter lysis buffer (Promega, Madison, WI), and lysates were collected. The β-galactosidase assay was performed following standard protocol (Promega) and a standard curve of 1 to 5 mU of β-galactosidase was run concurrently with the samples. The enzyme activity of each sample was normalized to the protein concentration of each cell lysate as measured by the BCA protein assay (Pierce, Rockford, IL). In each experiment, lysates were collected from cultured MEFs from nontransgenic mice to serve as the baseline indicator of Lac Z activity, and the activity of each promoter construct is expressed relative to control activity. All activities represent at least three independent experiments, with MEFs from each construct tested in triplicate per experiment. Relative Lac Z activities are expressed as the mean ± SD computed from the results obtained from each experiment.

Results

α-SMA Promoter Region from −2600 through the First Intron Conferred in Vivo *Expression of a Lac Z Reporter in Myofibroblasts in Granulation Tissue*

Previous results using transgenic animals demonstrated that the first intron along with −2.6 kb of the α-SMA promoter was required for the transgene construct to be expressed in SMCs in embryos and adults in a pattern that closely mimicked endogenous α-SMA expression.[15] Using these same transgenic mice we examined whether this promoter region could drive transgene expression in myofibroblasts in granulation tissue and whether the pattern of transgene expression mimicked expression of α-SMA in the myofibroblasts. Granulation tissue was examined at 7, 10, 14, and 21 days after wounding. At 7 days after wounding a well-formed granulation tissue was present (Figure 1A). Lac Z staining, demonstrating expression of the p2600Int/Lac Z transgene, could be observed in the granulation tissue (Figure 1, A and E). Myofibroblasts could be identified by their strong α-SMA immunostaining (Figure 1, C and G). Co-localization of Lac Z and α-SMA immunostaining could be observed in the myofibroblasts (purple color due to overlap of red α-SMA and blue Lac Z staining; Figure 1,

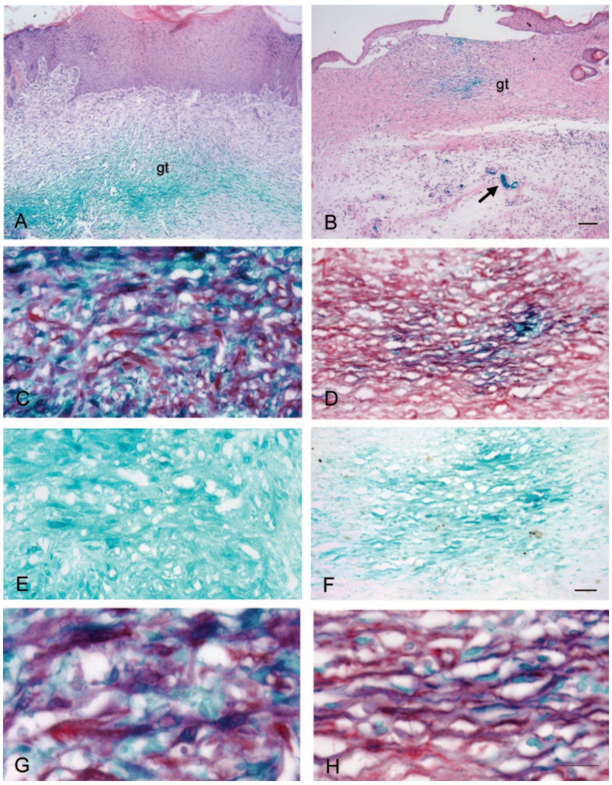

Figure 1. A–H: Histological examination of p2600Int/Lac Z expression in granulation tissue. Cutaneous wounds were made on the dorsum of transgenic mice, collected at 7 days (**A**, **C**, **E**, **G**) and 14 days (**B**, **D**, **F**, **H**) after wounding, stained for Lac Z, fixed, and embedded in paraffin. Sections were either counterstained with H&E (**A**, **B**), immunostained for α-SMA (**C**, **D**, **G**, **H**), or visualized for Lac Z staining only (**E**, **F**). **A** and **B:** Lac Z expression was seen in granulation tissue (gt) and in vascular SMCs (**arrows**). **C** and **D:** α-SMA-immunostained section adjacent to section in **A** or **B** at higher magnification of region with Lac Z-positive cells. Co-localization of Lac Z expression and α-SMA-positive myofibroblasts can be visualized by purple color due to overlap of red α-SMA staining and blue Lac Z staining. Compare with **E** and **F** that illustrate Lac Z blue staining only. **E** and **F:** Section adjacent to section in **C** or **D** illustrating Lac Z expression only. Note that Lac Z expression appears to be present in all α-SMA-containing myofibroblasts; however, Lac Z expression is reduced in many of the myofibroblasts at 14 days after wounding (**F**). **G** and **H:** Higher magnification of sections in **E** and **F** illustrating co-localization of α-SMA and Lac Z within the same cell. Scale bars: 100 μm (**A**, **B**); 25 μm (**C–F**); 20 μm (**G–H**).

C and G). All of the α-SMA-positive myofibroblasts at day 7 appeared to be positive for Lac Z staining. By 14 days after wounding there were fewer Lac Z-positive cells in the granulation tissue and they appeared to be more prevalent near the center of the wound (Figure 1B). Lac Z staining was present in α-SMA-positive myofibroblasts (Figure 1; D, F, and H); however, the staining for Lac Z appeared to be reduced (compare Figure 1, E and F). When Lac Z staining by itself was compared with α-SMA staining of an adjacent section the α-SMA-positive myofibroblasts appear to have Lac Z staining, although in some cells the staining was very reduced (Figure 1, D and F). By 21 days after wounding α-SMA-positive myofibroblasts had disappeared from the wound and the Lac Z staining was gone except for vascular SMCs (not illustrated).

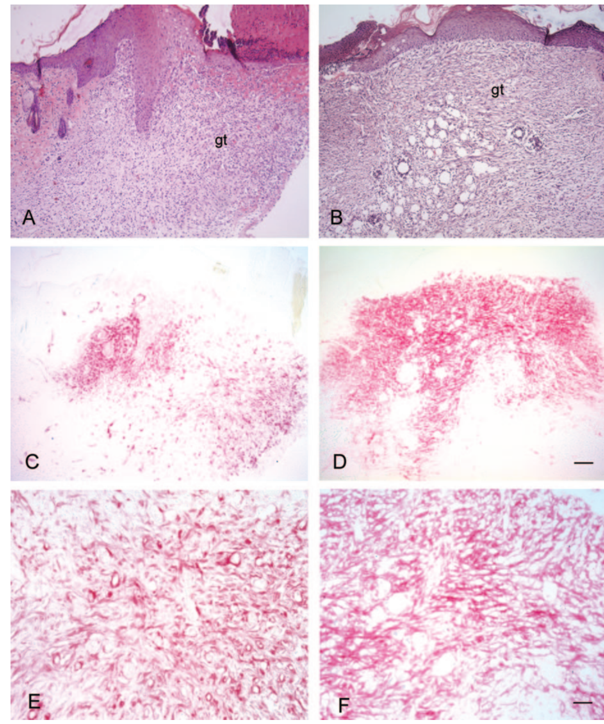

Figure 2. A–F: Effects of mutations to intronic CArG (**A**, **C**, **E**) or TCE (**B**, **D**, **F**) on the expression of the p2600Int/Lac Z transgene in granulation tissue. Cutaneous wounds were made on the dorsum of transgenic mice, collected at 7 days after wounding, stained for Lac Z, fixed, and embedded in paraffin. Sections were either counterstained with H&E (**A**, **B**) or immunostained for α-SMA (**C–F**). No Lac Z expression is observed in α-SMA-positive myofibroblasts in the granulation tissue (gt). Scale bars: 100 μm (**A–D**); 40 μm (**E**, **F**).

Mutation of the Intronic CArG Abolished Activity of the p2600Int/Lac Z Transgene in Myofibroblasts in Granulation Tissue

Previously it has been demonstrated that mutation of the intronic CArG abolishes the activity of the p2600Int/Lac Z transgene in SMCs in developing and adult mice.[15] Interestingly this mutation does not abolish activity in developing skeletal and cardiac muscle, where α-SMA is normally expressed during development before expression of the specific sarcomeric α-actin.[8,15] These results suggest differential molecular regulation of the α-SMA promoter in developing skeletal and cardiac muscle versus SMCs. To determine whether regulation of α-SMA promoter activity in myofibroblasts in granulation tissue is similar to developing sarcomeric muscle or to SMCs we generated transgenic mice containing the mutated intronic CArG p2600Int/Lac Z transgene. Three independent founders showed persistence of expression in skeletal and cardiac muscle during development and consistent with previous reports of Mack and Owens[15] none of these founders expressed Lac Z in SMC. The two transgenic mouse strains that demonstrated strong expression of the transgene in developing sarcomeric muscle were analyzed for activity of the promoter in myofibroblasts in granulation tissue. In contrast to the wild-type p2600Int/Lac Z transgene (Figure 1), no Lac Z activity was observed in granulation tissue myofibroblasts at any of the postwound stages examined in either of the trans-

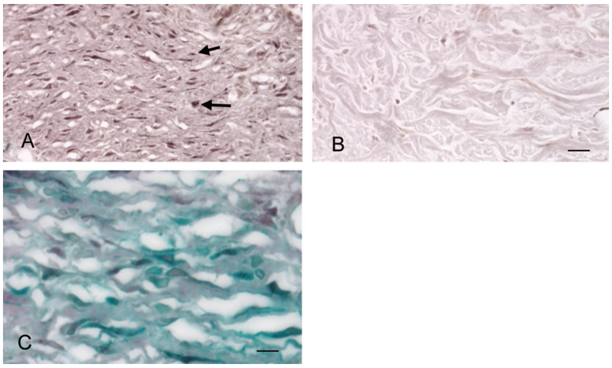

Figure 3. A–C: Immunolocalization of SRF in granulation tissue and dermis. Cutaneous wounds were made on the dorsum of either wild-type or transgenic mice carrying the p2600Int α-SMA promoter, collected at 7 days after wounding, fixed, and embedded in paraffin. Sections were immunostained for SRF. **A:** Myofibroblasts in granulation tissue demonstrated strong nuclear immunostaining for SRF (**arrows**). **B:** Little immunostaining for SRF, either nuclear or cytoplasmic, was observed in fibroblasts in dermis adjacent to the granulation tissue. **C:** Nuclear staining for SRF can be observed in cells in the granulation tissue expressing the p2600Int/Lac Z transgene. Scale bars: 25 μm (**A**, **B**); 15 μm (**C**).

genic strains containing the mutated intronic CArG p2600Int/Lac Z transgene (Figure 2; A, C, and E). These results suggest that regulation of the α-SMA promoter in granulation tissue myofibroblasts is different from the developing sarcomeric muscle and, like SM, requires the intronic CArG element for activity.

Nuclei of Myofibroblasts in Granulation Tissue Immunostain for SRF

SRF has been demonstrated to bind to the intronic CArG element in the α-SMA promoter and play a critical role in the activity of this promoter.[15,28] It has been demonstrated in myofibroblasts that form in the lung in response to bleomycin that SRF levels increase and that SRF immunoreactivity increases in the nucleus.[22] To determine whether nuclear SRF was increased within activated myofibroblasts, SRF immunostaining was compared between myofibroblasts within 7-day granulation tissue and adjacent normal dermal fibroblasts. SRF immunostaining was dramatically increased in the nuclei of myofibroblasts compared to dermal fibroblasts (Figure 3, A and B). SRF immunostaining was also co-localized with Lac Z in myofibroblasts in 7-day granulation tissue (Figure 3C). These results suggest that SRF is predominantly located in the nucleus in myofibroblasts in granulation tissue and that SRF is in the proper location to contribute to CArG-dependent expression of α-SMA in activated myofibroblasts.

Mutation of the TCE Abolished Activity of the p2600Int/Lac Z Transgene in Myofibroblasts in Granulation Tissue

The TCE within a 125-bp promoter context is required for TGF-β1 inducibility of α-SMA in cultured fibroblasts and SMCs.[16,17] To determine whether the TCE is required for expression of the p2600Int promoter in myofibroblasts in

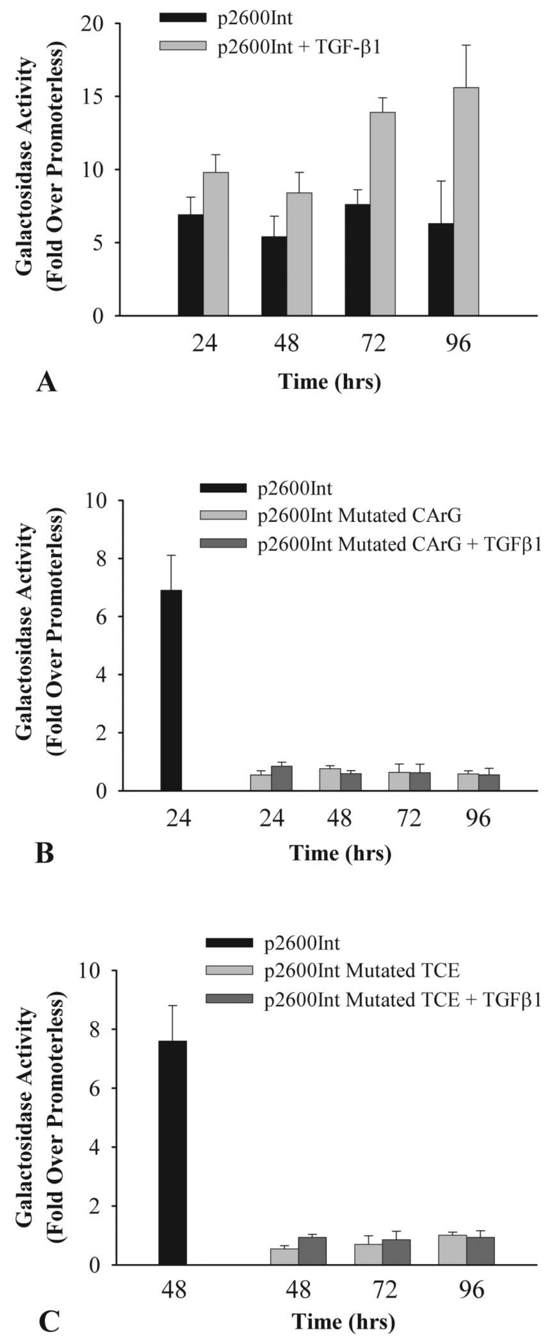

Figure 4. A–C: The effects of intronic CArG and TCE mutations on p2600Int/Lac Z activity in cultured fibroblasts. Mouse embryonic fibroblasts were obtained from mice carrying the p2600Int/Lac Z transgene (**A**) or transgene with a mutated intronic CArG (**B**) or TCE (**C**). After culture in the absence or presence of TGF-β1 for the indicated period of time, cells were lysed and β-galactosidase activity measured spectrophotometrically. Galactosidase activity (±SD) is expressed relative to the baseline galactosidase activity of mouse embryonic fibroblasts that do not carry the p2600Int/Lac Z transgene set to 1. **A:** The p2600Int/Lac Z transgene had significant transcriptional activity without TGF-β1 and this activity increased significantly with TGF-β1 treatment throughout time in culture. **B** and **C:** Mutation of the intronic CArG and the TCE greatly decreased activity and abolished responsiveness to TGF-β1. Similar low levels of activity were observed after mutation of either of these elements.

granulation tissue we obtained transgenic mice with a mutation in the TCE of the p2600Int/Lac Z transgene. Granulation tissue was examined at 7, 10, 14, and 21 days after wounding for co-localization of α-SMA-positive

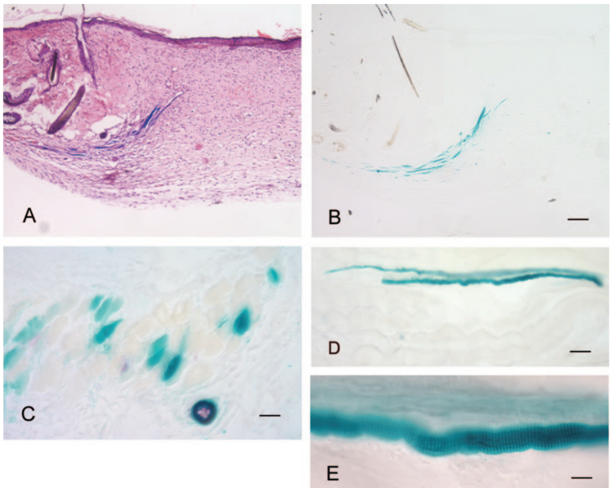

Figure 5. A–E: Expression of p2600Int/Lac Z transgene in the panniculus carnosus skeletal muscle layer. **A** and **B:** Cutaneous wounds were made on the dorsum of transgenic mice, collected at 14 days after wounding, stained for Lac Z, fixed, and embedded in paraffin. **C:** Some of the sections were immunostained for α-SMA. **D** and **E:** Normal skin was obtained from the dorsum of transgenic mice, stained for Lac Z, fixed, and examined as whole mounts. **A** and **B:** Strong expression of Lac Z was observed in panniculus carnosus muscle undergoing repair after cutaneous wounding as observed in both H&E-stained (**A**) and unstained (**B**) sections. **C:** At higher magnification Lac Z expression can be observed in both individual myotubes (**arrows**) and surrounding blood vessels. Note perivascular cells around blood vessels are purple indicative of both Lac Z and α-SMA expression whereas myotubes are only blue due to Lac Z expression. **D** and **E:** Occasional Lac Z-positive myotubes can be observed in whole mounts of normal panniculus carnosus. At higher magnification banding of myofibrils can be observed in Lac Z-positive myotube. Scale bars: 100 μm (**A**, **B**); 25 μm (**C**); 10 μm (**D–F**).

myofibroblasts and Lac Z expression. No Lac Z expression was observed in granulation tissue myofibroblasts at any of the postwound stages examined (Figure 2; B, D, and F). Similarly, no Lac Z staining was observed in vascular SMCs. These results demonstrate that both the TCE and the intronic CArG elements are required for activity of p2600Int promoter in myofibroblasts in granulation tissue.

Both the Intronic CArG and the TCE in the p2600Int α-SMA Promoter Are Required for TGF-β1 Responsiveness in Cultured Fibroblasts

TGF-β1 is proposed to play an important role in promoting the expression of α-SMA and the differentiation of myofibroblasts in granulation tissue.[1,5,10] We demonstrated in the experiments described above that both the intronic CArG and the TCE in the α-SMA promoter are critical for its activity in myofibroblasts in granulation tissue. To determine directly whether these elements are required for responsiveness of the p2600Int α-SMA promoter to TGF-β1 we isolated fibroblasts from transgenic embryos carrying the p2600Int/Lac Z transgene, the mutated intronic CArG p2600Int/Lac Z transgene, or the mutated TCE p2600Int/Lac Z transgene. These fibroblasts were cultured in the absence or presence of TGF-β1 and analyzed for the expression of the transgene. Fibroblasts containing the p2600Int/Lac Z transgene and cultured in the absence of TGF-β1 expressed the transgene at a basal level throughout the 96 hours measured (Figure 4A). TGF-β1 treatment increased the activity of the transgene within 24 hours above the basal level and the activity continued to increase for the 96 hours measured (Figure 4A). In contrast, there was essentially no increase in expression of the mutated intronic CArG p2600Int/Lac Z transgene in cultured fibroblasts on addition of TGF-β1 (Figure 4B). The basal level of transgene expression observed in fibroblasts containing the p2600Int/Lac Z transgene was also abolished by mutation of the intronic CArG element (Figure 4B). Similarly, there was essentially no expression of the mutated TCE p2600int/Lac Z transgene in cultured fibroblasts in the presence or absence of TGF-β1 (Figure 4C). Interestingly, the very low level of expression of the mutated CArG and the mutated TCE p2600Int/Lac Z transgene was similar. These results demonstrate that both the intronic CArG and the TCE are essential for activity of the p2600Int/Lac Z transgene in myofibroblasts in granulation tissue and both are required for responsiveness to TGF-β1.

p2600Int/Lac Z Transgene Is Expressed in Adult and Regenerating Panniculus Carnosus Skeletal Muscle in Dermal Wounds and Abolished by Mutation to Intronic CArG and TCE

During embryonic development of skeletal muscle α-SMA is expressed for a short period of time before the appearance of the skeletal α-actin and the p2600Int/Lac Z transgene is also active during this time.[8,15] Mice contain a thin layer of skeletal muscle in their dermis termed the panniculus carnosus. When making a full-thickness cutaneous wound the panniculus carnosus is cut and this muscle layer repairs itself during wound healing and comes to underlie the newly formed scar.[29] Lac Z staining, indicative of activity of the p2600Int/Lac Z transgene, was observed in myotubes in the panniculus carnosus near the wound margin (Figure 5; A to C). Although there is strong Lac Z staining indicative of promoter activity, there is essentially no immunostaining for α-SMA in these cells (Figure 5C). Because of these results we examined whether the p2600Int/Lac Z transgene is active during the normal process of muscle repair. Whole mounts of normal dermis revealed that there were occasional myotubes that stained for Lac Z (Figure 5, D and E). The panniculus carnosus skeletal muscle undergoing repair after cutaneous wounding of transgenic animals containing either mutations in the CArG or the TCE of the p2600Int/Lac Z transgene was examined for Lac Z expression. No Lac Z staining was observed in any of the regenerating skeletal muscle examined in either strain of transgenic mouse (not illustrated). These results are different from what is observed in developing skeletal muscle and suggests that regulation of α-SMA expression is different during development and repair of skeletal muscle (Table 1).

Table 1. Evaluation of Transgene Expression

	Myofibroblasts	Vascular SM cells	Developing skeletal muscle	Regenerating skeletal muscle
P2600Int	+	+	+	+
Mutated intronic CArG	−	−	+	−
Mutated TCE	−	−	−	−

The listed tissues from transgenic mice carrying the appropriate transgene were evaluated for transgene expression and listed as + if Lac Z expression was observed and − if no Lac Z expression was observed.

Discussion

The proper formation and function of myofibroblasts during wound healing is critical for wound closure.[1,2] Although numerous studies have examined the *cis*-elements and *trans*-acting factors that regulate expression of α-SMA and other SM-specific proteins in SMCs, little is known about the regulation of α-SMA in myofibroblasts in granulation tissue. In this study we demonstrated that, similar to SMCs, a promoter sequence containing −2600 through the first intron of the α-SMA gene would promote expression of α-SMA in myofibroblasts in granulation tissue. We also provided clear evidence that α-SMA expression in myofibroblasts in granulation tissue is dependent on the intronic CArG element and the TCE and that both of the elements are required for TGF-β1 inducibility of α-SMA expression. Last, we found that the p2600Int α-SMA promoter is active in repairing skeletal muscle and that unlike during development both the intronic CArG and the TCE are required for α-SMA promoter activity in skeletal muscle undergoing repair. These studies demonstrate that regulation of expression of α-SMA is similar in SMCs, myofibroblasts, and regenerating skeletal muscle with regard to the necessity of the intronic CArG and TCE (Table 1); however, it should be stressed that there must be cell-specific regulation of α-SMA expression in these cells. As such, the results of the present study represent a key first step in efforts to identify mechanisms that control cell-selective expression of α-SMA within myofibroblasts in granulation tissue and to begin to identify regulatory pathways and environmental cues that regulate this process.

Our findings clearly demonstrate that the sequence of the α-SMA promoter from −2600 through the first intron is sufficient to drive high-level expression of the transgene in myofibroblasts in granulation tissue. Transient transfection analysis has previously demonstrated that the first 125 bp of the α-SMA promoter were sufficient for promoter expression in cultured fibroblasts;[17,18] however, inclusion of regions upstream from −125 up to −2.8 kb repressed promoter expression suggesting that positive-acting *cis*-elements located outside −2.8 kb of the α-SMA promoter were required for expression.[17] Our results are consistent with the hypothesis that the first intron in the α-SMA gene contains sequences essential for α-SMA promoter activity. The CArG A, CArG B, and intronic CArG elements within this portion of the promoter have all been demonstrated to be necessary for full expression of α-SMA in SMCs. We chose to examine intronic CArG in this study since it has been demonstrated to regulate cell-type-specific expression in SMCs and developing skeletal and cardiac muscle and has not been previously examined in myofibroblasts.[15] Mutation of the intronic CArG element abolished expression of the p2600Int promoter in myofibroblasts in granulation tissue, as well as abolished basal expression in cultured fibroblasts and responsiveness to TGF-β1. These results demonstrate that, similar to SM cells, the intronic CArG element in the p2600Int α-SMA promoter sequence is essential for activity in myofibroblasts in granulation tissue and may be the essential sequence outside −2.8 kb of the α-SMA promoter required for expression in myofibroblasts.

SRF is a relatively ubiquitous transcription factor that can bind the CArG elements in the α-SMA promoter.[15,19] We demonstrate here that, unlike dermal fibroblasts, which show little immunostaining for SRF, the nuclei of myofibroblasts in granulation tissue stain intensely for SRF. SRF expression and nuclear localization has also been demonstrated to increase in lung myofibroblasts in a rat bleomycin fibrosis model.[22] Localization of SRF to the nucleus in myofibroblasts is consistent with its putative role of regulating the expression of SM-specific cytoskeletal proteins in SM cells.[14,15,19]

TGF-β1 is believed to play a critical role in promoting expression of α-SMA and myofibroblast differentiation both *in vitro* and *in vivo.*[1,5,10] We have demonstrated here for the first time that the TCE is essential for α-SMA activity in myofibroblasts in granulation tissue. Because the TCE is a non-Smad-binding element,[16] these results suggest that TGF-β1 can promote α-SMA expression and myofibroblast differentiation by activating a non-Smad pathway. Previously, we have also found that TGF-β1 can promote α-SMA expression and increased force generation in Smad-3 knockout fibroblasts, consistent with a Smad-independent pathway promoting myofibroblast differentiation (J.J. Tomasek and colleagues, unpublished results). In addition to the TCE, we also found that the intronic CArG element is essential for TGF-β1 inducibility of α-SMA. These results suggest that these two elements operate interactively rather than independently to allow expression of α-SMA in myofibroblasts in granulation and increased expression in response to TGF-β1.

In the course of this study we observed that as the panniculus carnosus underwent repair after cutaneous wounding there was strong staining for Lac Z in myotubes demonstrating activity of the p2600Int/Lac Z transgene. A previous study has reported that after cutaneous wounding of neonatal rats there was intermittent immunostaining for α-SMA in myotubes adjacent to the wound edge and that this staining decreased in adult rats.[30] We also observed very little immunostaining for α-SMA in myotubes undergoing repair in cutaneous wound tissue of adult mice, despite the strong staining for Lac Z. It is not clear as to why there is such strong activity of the p2600Int α-SMA promoter with little immunostaining for α-SMA protein; it may be that there is posttranscriptional regulation decreasing α-SMA synthesis, alternatively the epitope for the anti-α-SMA antibody may be masked in these cells. The regulation of the expression of α-SMA appears to be different during development of skeletal muscle and during repair, with both the CArG and TCE necessary for expression during repair but not development (Table 1). Whether this difference may be due to the different mechanisms by which skeletal muscle regulates α-SMA expression during development versus repair remains to be examined.

In summary, these studies are the first to describe sufficient promoter-enhancer regions of the α-SMA gene that are required to drive expression of α-SMA in myofibroblasts in granulation tissue. Moreover, these results provide novel evidence that activation of this gene in myofibroblasts *in vivo* is dependent on both an intronic CArG and TCE, similar to that observed in SM. We also found that the intronic CArG element was dispensable for α-SMA promoter activity in developing skeletal muscle but necessary for transgene activation in skeletal muscle undergoing repair. As such, these studies add to a growing body of evidence demonstrating that although multiple cell types can express the α-SMA gene under different circumstances, certain aspects of regulation are cell-type selective and, that similar to SM cells,[14] α-SMA expression in myofibroblasts is not controlled by a few SM cell-specific transcription factors but rather by complex combinatorial interactions between multiple general and tissue-specific proteins. Finally, these results provide a foundation for further investigation of molecular mechanisms, signal transduction pathways, and environmental cues that regulate the activity of the intronic CArG and TCE elements and thereby contribute to myofibroblast activation. Future studies are needed to elucidate these control processes as well as to determine what elements within the α-SMA promoter confer myofibroblast-specific expression and how myofibroblasts express only a subset of SM-specific cytoskeletal proteins.

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